Efficacy of Shoushen granule on adenosine triphosphate binding cassette transporter A1, proprotein convertase subtilisin/kexin type 9 and toll-like receptor 4/nuclear factor kappa-B signaling pathway in ApoE-knockout mice

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Abstract

OBJECTIVE: To evaluate the efficacy of Shoushen granule, prepared with four Chinese medicinal herbs, on the targeted regulation of adenosine triphosphate binding cassette transporter A1 (ABCA1) through proprotein convertase subtilisin/kexin type 9 (PCSK9) and toll-like receptor 4 (TLR4) / nuclear factor kappa-B (NF-κB) signaling pathways involved in affecting atherosclerosis (AS) in ApoE-knockout (ApoE-/-) mice.

METHODS: ApoE-/- mice fed with a high-fat diet were used for AS modeling and divided into Model, Shoushen, and Atorvastatin groups. C57BL/6J mice at the same age and background strain were included in the Control group. Western blot and immunohistochemistry were used to measure ABCA1, PCSK9, TLR4, and NF-κB protein expression in mouse aortas. Enzyme-linked immuno sorbent assay was used to measure mouse serum tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), monocyte chemotactrant protein 1 (MCP-1), and intercellular cell adhesion molecule-1 (ICAM-1) expression. Serum lipid profiles and histopathology were also assessed. Shoushen granule were composed of Heshouwu (Radix Polygoni Multiflori) 15 g, Gouqizhi (Fructus Lycii) 15 g, Sheng shanzha (Raw Fructus Crataegus Pinnatifidae) 10 g, and Sanqi (Radix Notoginseng) 3 g.

RESULTS: ApoE-/- mice fed with a high-fat diet had notable AS lesions, with reduced ABCA1 and IL-10 levels, elevated PCSK9, TLR4, NF-κB, TNF-α, MCP-1, and ICAM-1 expression, and increased total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) contents. With drug interventions, the areas of AS plaques were significantly reduced, the ABCA1 and IL-10 levels were increased, while the PCSK9, TLR4, NF-κB, TC, and LDL-C contents, and the TNF-α, MCP-1, and ICAM-1 expression were reduced.
CONCLUSION: Shoushen granule effectively interfered with AS development by antagonizing the expression of key factors of the PCSK9 and TLR4/NF-κB signaling pathway to upregulate ABCA1 expression.

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Keywords: Atherosclerosis; ATP-binding cassette transporters; Proprotein convertases; Toll-like receptor 4; NF-kappa B; Shoushen granule

INTRODUCTION
Atherosclerosis (AS) is the pathological basis of cardiovascular and cerebrovascular diseases, and is closely related to aging and senescence. Abnormal lipid metabolism plays an important role in AS. Previous study has shown that adenosine triphosphate binding cassette transporter A1 (ABCA1) promotes cholesterol efflux of macrophages and plays an important role in AS development and progression. Human proprotein convertase subtilisin/kexin type 9 (PCSK9) directly downregulates ABCA1 protein expression to mediate cholesterol efflux, which is closely associated with AS development and progression. Toll-like receptors (TLRs), in particular Toll-like receptor 4 (TLR4), have a close relationship with AS and play an important role in its progression. Nuclear factor kappa-B (NF-κB) plays an important role in vivo immune responses, inflammatory responses, cell growth, and development through the initiation and regulation of gene transcription, thereby directly or indirectly being involved in AS development and progression. NF-κB target genes tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) are involved in the inflammatory response during AS development. Monocyche chemotactic protein (MCP-1) accelerates the formation of foam cells and promotes AS development and progression. Intercellular adhesion molecule (ICAM-1) is highly expressed by stimulation from inflammatory cytokines to enhance adhesion between monocytes and endothelial cells, promoting inflammation and initiating AS progression. Our previous studies showed that the kidney-invigorating compounded Chinese medicine formula, Shoushen granule, effectively treat AS.

In this study, the aim was to evaluate the efficacy of Shoushen granule on the targeted regulation of ABCA1 through PCSK9 and TLR4/NF-κB signaling pathway, and to reveal the possible mechanism underlying Shoushen granule effect on AS treatment.

MATERIALS AND METHODS

Experimental animals
Twelve-week male ApoE−/− mice under specific pathogen-free (SPF) conditions as well as male C57BL/6J mice at the same age and background strain, three-month-old, weighing (20 ± 5) g, were purchased from Shanghai Biomodel Organism Science & Technology Development Co., Ltd. (Shanghai, China, animal license No. SCXK (Shanghai) 2014-0002). Animals were housed and fed individually in single cages in the animal facilities of Shanghai Biomodel Organism Science & Technology Development Co., Ltd. under SPF conditions, with the temperature and humidity ranging from 19–22 °C and 50%-70%, respectively. Breeding conditions and experimental procedures of all animals strictly followed the rules and regulations of the Experimental Animal Ethics Committee of Shanghai Biomodel Organism Science & Technology Development Co., Ltd.

Drugs and reagents
Shoushen granule (Jiangyin Tianjiang Pharmaceutical Co., Ltd., Jiangsu Province, China) are composed of Heshouwu (Radix Polygoni Multiflori) 15 g, Gouqizi (Fructus Lycii) 15 g, Sheng shanzha (Raw Fructus Crataegi Pinnatifidae) 10 g, and Sanqi (Radix Notoginseng) 3 g. Atorvastatin tablets (Pfizer Newyork, US), mouse anti-ABCA1 antibody (Thermo Fisher Scientific, Massachusetts, US), rabbit anti-PCSK9 antibody (Abcam, Cambridge, UK), rabbit anti-TLR4 antibody (Abcam, Cambridge, UK), rabbit anti-NF-κB antibody (Cell Signaling Technology, Boston, US), rabbit anti-β-actin antibody (Cell Signaling Technology, Boston, US), goat anti-rabbit fluorescence secondary antibody (LI-COR, Nebraska, US), goat anti-mouse fluorescence secondary antibody (LI-COR, Nebraska, US), mouse total cholesterol (TC) enzyme-linked immunosorbent assay (ELISA) kit (EK-Bioscience, Shanghai, China), mouse triglyceride (TG) ELISA kit (EK-Bioscience, Shanghai, China), mouse high density lipoprotein-cholesterol (HDL-C) ELISA kit (EK-Bioscience, Shanghai, China), mouse low density lipoprotein-cholesterol (LDL-C) ELISA kit (EK-Bioscience, Shanghai, China), mouse tumor necrosis factor alpha (TNF-α) ELISA kit (EK-Bioscience, Shanghai, China), mouse IL-10 ELISA kit (EK-Bioscience, Shanghai, China), mouse monocyte chemoattractant protein 1 (MCP-1) ELISA kit (EK-Bioscience, Shanghai, China), mouse intercellular adhesion molecule 1 (ICAM-1) ELISA kit (EK-Bioscience, Shanghai, China), PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific, Massachusetts, US), biocytinonic acid (BCA) protein concentration determination kit (Beyotime Institute of Biotechnology, Shanghai, China), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel preparation kit (Beyotime Institute of Biotechnology, Shanghai, China) were drugs and key reagents used in this study.

AS modeling and grouping
A total of 72 12-week male ApoE−/− mice were fed with...
regular feed during the 1-week adaptive period, followed by random number table method into three groups: ApoE−/− mice fed with high-fat diet group (Model group, n = 24), ApoE−/− mice fed with high-fat diet and Shoushen granule (Shoushen group, n = 24), and ApoE−/− mice fed with high-fat diet and atorvastatin (Atorvastatin group, n = 24). Male C57BL/6J mice at the same age and background strain were used as the normal Control group (Control group, n = 24). The high-fat diet formula included regular mouse feed with additional fat (21%) and cholesterol (0.5%). Animals in the Shoushen and Atorvastatin groups were administered Shoushen granule and atorvastatin aqueous solution by oral gavage. Specific dosage was in accordance with the experimental methodology of herbal pharmacology. According to the equivalent dose calculation for mouse based the 60-kg bodyweight of an adult human, the gavage doses of the Shoushen and Atorvastatin groups were 25 and 4 mg/kg once per day for a total of 12 weeks, respectively. Animals in the control and Model groups were given an equal volume of distilled water by oral gavage once per day for a total of 12 weeks.

**General morphology in mice**

The body size, hair color, behavioral response, and feeding conditions of each mouse were recorded. In addition, each mouse was weighed every two weeks.

**Histopathological changes in mouse aortas observed under light microscopy**

The aortic arch located 0.5 cm away from the aortic root of each tissue sample was collected and fixed with 4% paraformaldehyde, then dissected and dehydrated. Part of the aortic arch was paraffin-embedded and serially sectioned (5 µm thickness) from the aortic root for HE and Masson’s staining, and the other part of the aortic arch was embedded in optimal cutting temperature (OCT) compound and serially sectioned (10 µm thickness) from the aortic root for Oil red O staining. An Olympus BH2 series polarized light microscope (Olympus, Tokyo, Japan) and photographed using a Nikon 4500 digital camera (Nikyo, Tokyo, Japan) for further semi-quantitative analysis. Semi-quantitative analysis was conducted as follows: Average optical density (AOD) = OD in the area with positive expression × percentage of area with positive expression (area%).

**Western blot for the detection of ABCA1, PCSK9, TLR4, and NF-κB protein expression**

Total protein extracted from mouse aortas was quantified by BCA, followed by separating the protein in SDS-PAGE and wet transfer to a PVDF membrane, which was blocked with 5% bovine serum albumin for 1 h and incubated with primary antibody working solution (1: 2000 mouse anti-ABCA1 antibody, 1: 1000 rabbit anti-PCSK9 antibody, 1: 1000 rabbit anti-TLR4, and 1: 1000 rabbit anti-NF-κB antibody) individually at 4 °C overnight. After washing 3 times (5 min each), the PVDF membrane was incubated with the corresponding secondary antibody (either goat anti-rabbit fluorescence secondary antibody or goat anti-mouse fluorescence secondary antibody) at room temperature for 1 h. An Odyssey far-infrared luminescence scanner (Li-cor, Nebraska, US) was used to capture the image, followed by using Image J software (Image J, Rawak software, Inc. Germany) to analyze the gray value of the protein bands. Relative target protein content was calculated according to the gray value of the target band/the gray value of the β-actin band.

**ELISA for the detection of mouse serum TNF-α, IL-10, MCP-1, and ICAM-1 levels**

Serum of each mouse was collected as described in the previous subsection. Serum TNF-α, IL-10, MCP-1, and ICAM-1 levels were measured strictly in accordance with the manufacturer’s instructions with the ELISA kits based on the OD of the tested samples, followed by preparing the corresponding standard curves and looking for the corresponding concentration ranges according to the standard curves.
the data. Independent sample t test or one-way analysis of variance was used to compare the differences between groups. SPSS 17.0 software (SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago, IL, USA) was used for statistical analysis. Graph Pad Prism 5 Project software (Graph Pad, California, USA) was used for graph preparation. P < 0.05 was the statistically significant level.

RESULTS

General conditions of the mice
During the experimental intervention, the control mice had well-proportioned limbs and shiny hair, and were quick and agile. Compared with the Control group, mice in the Model group were obese with dull hair and slow movement. Mice in the Shoushen and the Atorvastatin groups had relatively well-proportioned limbs, and fairly shiny hair, and were rather quick and agile. Animals in each group were well fed. Dynamic detection of mouse bodyweight showed no significant difference in bodyweight between different groups at 12 weeks of age (P > 0.05). Increase in bodyweight of all animals at 12-16 weeks of age and in different groups was consistent. At 16 weeks of age, the bodyweight of mice in the Control group was significantly different from those in the Model, Shoushen, and Atorvastatin groups (P < 0.05). No significant difference in bodyweight was found between the Model, Shoushen, and Atorvastatin groups (P > 0.05). At 18 weeks of age, the bodyweights of mice in the Shoushen and Atorvastatin groups was steadily increased, and were not significantly different from the control and Model groups (P > 0.05), while bodyweights of the control and the Model groups were significantly different at this age (P < 0.05). At 18-24 weeks of age, bodyweights of the Shoushen and Atorvastatin groups were steadily reduced, and until 24 weeks of age, bodyweights of the Model group were significantly higher than the control, Shoushen, and Atorvastatin groups (P < 0.05). No significant differences in bodyweight were found between the control, Shoushen, and Atorvastatin groups at 24 weeks of age (P > 0.05, Figure 1).

Lipid profile results in different groups of mice
In the Model group, serum TC and LDL-C contents were significantly elevated, and serum HDL-C content was significantly reduced, while in the Shoushen and Atorvastatin groups, serum TC and LDL-C contents were significantly lower than in the Model group (P < 0.05 and P < 0.01, respectively), and no significant difference in HDL-C content was found between the Shoushen and Atorvastatin groups (P > 0.05). No significant differences in TC and LDL-C were found between the Shoushen and Atorvastatin groups (P > 0.05). No significant difference in TG content was found between different groups (P > 0.05, Table 1).

![Figure 1 Detection results of dynamic body weight on mice](image)

Data are presented as mean ± standard deviation. *P < 0.05, compared to Control group, Shoushen group and Atorvastatin group.

| Table 1 Comparison of blood lipids in each group (nmol/L, ± s) |
|---|---|---|---|---|
| Group | n | TC | TG | HDL-C | LDL-C |
| Control | 12 | 6.18±0.70 | 5.87±0.36 | 3.98±0.23 | 2.48±0.62 |
| Model | 12 | 6.89±0.43 a | 6.10±0.22 | 3.71±0.28 a | 3.25±0.69 a |
| Shoushen | 12 | 6.20±0.42 b | 5.86±0.25 | 3.90±0.23 | 2.55±0.70 b |
| Atorvastatin | 12 | 6.18±0.46 | 5.88±0.32 | 3.96±0.25 | 2.53±0.52 |

Notes: control: male C57BL/6j mice fed with normal diet; model: ApoE−/− mice fed with high-fat diet; Shoushen: ApoE−/− mice fed with high-fat diet and Shoushen granule; atorvastatin: ApoE−/− mice fed with high-fat diet and atorvastatin. TC: total cholesterol; TG: triglyceride; HDL-C: high density lipoprotein-cholesterol; LDL-C: low density lipoprotein-cholesterol. Data represent the mean ± standard deviation from 3 independent experiments. *P < 0.05, compared to control; *P < 0.01, compared to model.
Serum TNF-α, IL-10, MCP-1, and ICAM-1 expression in different groups of mice
The serum level of IL-10 in the Model group was significantly lower than that in the Control group ($P < 0.01$), TNF-α, MCP-1, and ICAM-1 levels of the Model group were significantly elevated ($P < 0.01$). Serum IL-10 level of the Shoushen and Atorvastatin groups significantly lower than the Model group ($P < 0.05$), TNF-α, MCP-1, and ICAM-1 levels were significantly lower than the Model group ($P < 0.01$). No significant differences in serum TNF-α, IL-10, MCP-1, and ICAM-1 levels were found between the Shoushen and Atorvastatin groups ($P > 0.05$, Table 2).

Hematoxylin and eosin (HE) staining of aortic tissues
AS plaque areas in the aortic roots of the Model group were significantly increased. With the Shoushen and Atorvastatin interventions, AS plaque areas in the aortic roots of mice were significantly reduced ($P < 0.05$ and $P < 0.01$, respectively). No significant difference in AS plaque areas was found between Shoushen and Atorvastatin groups ($P > 0.05$, Figure 2).

Oil red O staining of aortic tissues
The amounts of plaque and Oil red O stained lipid droplets in the aortic roots of the Control group were small, while the thickness of the endometrium in the aortic roots of the Model group was increased together with plaque formation and massive lipid infiltration in plaques stained with Oil red O. Relative lipid areas in the plaques of the Shoushen and Atorvastatin groups were smaller than in the Model group ($P < 0.01$), and no significant difference was found between the Shoushen and Atorvastatin groups ($P > 0.05$, Figure 2).

Masson’s trichrome staining of Aortic Tissues
The aortic walls of the Control group had a small amount of collagen fibers and smooth muscle fibers. AS plaques of the Model group had relatively thin fibrous gaps and less matrix-fiber composition. Matrix-fiber compositions of the fibrous gaps in AS plaques of the Shoushen and Atorvastatin groups were increased and evenly distributed, and no significant difference was found between them ($P > 0.05$, Figure 4).

ABCA1, PCSK9, TLR4, and NF-κB protein expression
ABCA1 protein expression in aortas of the Model group was significantly lower, while PCSK9, TLR4 ($P < 0.01$), and NF-κB expressions in the aorta were significantly higher than in the Control group ($P < 0.01$). ABCA1 protein expression in aortas of the Shoushen and Atorvastatin groups was significantly higher than in the Model group ($P < 0.01$), while PCSK9 expression in mouse aortas of the Shoushen and Atorvastatin groups was significantly lower than in the Model group ($P < 0.01$). No significant differences in the expression of these proteins were found between the Shoushen and Atorvastatin groups ($P > 0.05$, Figure 5).

Western blot analysis showed that in the Control group, ABCA1 protein expression in mouse aortas was relatively high, and PCSK9, TLR4, and NF-κB protein expressions in mouse aortas were relatively low. ABCA1 protein expression in aortas of the Model group was significantly lower than in the Control group ($P < 0.01$), and PCSK9, TLR4, and NF-κB protein expressions in aortas of the Model group were significantly higher than in the Control group ($P < 0.01$). Compared with the Model group, ABCA1 protein expression in aortas of the Shoushen and Atorvastatin groups was significantly elevated ($P < 0.01$), while PCSK9, TLR4, and NF-κB protein expression in aortas of the Shoushen and Atorvastatin groups was significantly reduced ($P < 0.01$). No significant differences in the expression of these proteins were found between the Shoushen and Atorvastatin groups ($P > 0.05$, Figure 6).

DISCUSSION
In this study we used 12-week old ApoE−/− mice fed with a high-fat diet as an in vivo AS model. Pathological findings of animal aortas showed obvious formation of AS plaques, lipid deposition, and reduction of collagen fibers. In addition, the serum lipid profile showed a significant increase of TC and LDL-C contents and decrease of HDL-C content in this mouse model. AS is a chronic and systemic degenerative disease characterized by lipid metabolism disorders.12 ABCA1, as an integral membrane protein, plays an important role in the efficient elimination of excess cholesterol in cells through ATP consumption and promoting reverse cholesterol transport.13-15 ABCA1 expression is positively correlated with the cholesterol content in macrophages and fibroblasts.13 Increased ABCA1 expression pro-

Table 2 Comparison of TNF-α, IL-10, MCP-1 and ICAM-1 detection in each group (nmol/L ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>TNF-α ± s</th>
<th>IL-10 ± s</th>
<th>MCP-1 ± s</th>
<th>ICAM-1 ± s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>424±16</td>
<td>335±16</td>
<td>16±1</td>
<td>302±17</td>
</tr>
<tr>
<td>Model</td>
<td>12</td>
<td>448±22</td>
<td>296±13</td>
<td>17±1</td>
<td>324±18</td>
</tr>
<tr>
<td>Shoushen</td>
<td>12</td>
<td>426±15</td>
<td>334±40</td>
<td>16±1</td>
<td>304±9</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>12</td>
<td>426±26</td>
<td>333±30</td>
<td>16±1</td>
<td>303±10</td>
</tr>
</tbody>
</table>

Notes: control: Male C57BL/6J mice fed with normal diet; model: ApoE−/− mice fed with high-fat diet; Shoushen: ApoE−/− mice fed with high-fat diet and Shoushen granule; atorvastatin: ApoE−/− mice fed with high-fat diet and atorvastatin. TNF-α: tumor necrosis factor alpha; IL-10: interleukin-10; MCP-1: monocyte chemoattractant protein 1; ICAM-1: intercellular adhesion molecule 1. Data represent the mean ± standard deviation from 3 independent experiments. *$P < 0.01$*, †$P < 0.001$, compared to control; ‡$P < 0.01$, compared to model.
motes HDL-C expression levels in the serum and liver of ABCA1-overexpressing transgenic mice to increase the cholesterol efflux of foamy macrophages, thereby reducing AS incidence.\textsuperscript{16,17}

PCSK9 is a subtilisin that regulates lipid metabolism and directly acts on the vascular wall. It is also expressed in AS plaques of human aortas and livers of ApoE\textsuperscript{-/-} mice.\textsuperscript{18} A previous study suggested that PCSK9 promotes LDL receptor degradation in hepatocytes to regulate lipid metabolism, thereby affecting serum LDL-C levels.\textsuperscript{19} PCSK9 also promotes lipid accumulation and inflammatory cytokine expression in macrophages by activating the TLR4/NF-κB signaling pathway.\textsuperscript{20} PCSK9 inhibitors promote ABCA1 protein expression and accelerate cholesterol efflux, thereby playing a role in anti-AS.\textsuperscript{21}

TLR4 is mainly expressed in AS vascular endothelial cells, smooth muscle cells, and macrophages, and is especially highly expressed in vascular endothelial cells in AS.\textsuperscript{22} A previous study has shown that TLR4 pathway-related gene expression and AS incidence of ApoE\textsuperscript{-/-} mice were higher than in C57BL/6J control mice of the same strain background.\textsuperscript{23} Activation of the TLR4 pathway through the MyD88-dependent signal transduction pathway activates the transcription factor NF-κB, which initiates transcription and synthesis of a variety of inflammatory cytokines such as MCP-1 and ICAM-1 in AS vascular endothelial cells and plaques, it also promotes foam cell formation of macrophages to inhibit cholesterol efflux and promote AS plaque development.\textsuperscript{24}

NF-κB is a transcription factor with many regulatory
roles in gene transcription and is closely associated with inflammatory responses, immune responses, cell proliferation, cell transformation, apoptosis, and other key pathophysiological processes. In this study, western blot analysis and IHC were used to measure the ABCA1, PCSK9, TLR4, and NF-κB protein expression in mouse aortas, and the results showed that a high-fat diet inhibited ABCA1 protein expression in aortas of ApoE−/− mice to induce PCSK9, TLR4, and NF-κB protein expression.

TNF-α is a major proinflammatory cytokine in AS plaques. It activates inflammatory cells, prevents extracellular matrix synthesis, and aggravates the thinning of fibrous gaps in plaques, thereby affecting plaque stability. Through the NF-κB pathway, TNF-α downregulates ABCA1 expression in macrophages to reduce cholesterol efflux. IL-10 is an anti-inflammatory cytokine with a variety of biological effects that inhibit adhesion and infiltration of inflammatory cells, such as monocytes and lymphocytes. In addition, it inhibits various inflammatory mediators to induce cytokine synthesis and secretion. MCP-1 is secreted by monocytes or macrophages. It is a proinflammatory cytokine with chemotactic and activating effects on monocytes, recruiting inflammatory cells to lesions of injury and in response to the stimulation of inflammatory cytokines. In addition, it activates leukocytes and mediates their production of inflammatory mediators to induce endothelial cell migration and smooth muscle cell division in plaques. ICAM-1 is a member of the immunoglobulin superfamily and an important member of the adhesion molecule family, and is mainly expressed in the vascular endothelial cells. ICAM-1 expression in the active endothelial cells is a key factor in tissue damage and inflammation caused by the aggregation and infiltration of circulating leukocytes. Inhibition of ICAM-1 expression reduces inflammatory cytokine aggregation and the adhesion of inflammatory cells and endothelial cells during AS formation, delaying AS progression. In this study, we used ELISA to detect serum TNF-α, IL-10, MCP-1, and ICAM-1 levels, and showed that a high-fat diet induced an increase in serum TNF-α, MCP-1, and ICAM-1 levels and a decrease in serum IL-10 levels in ApoE−/− mice.

Based on AS pathological features, our laboratory has studied the close relationship between AS, aging, and related degenerative diseases. In a series of study where herbal formulas for kidney invigoration were used to treat AS, we find that kidney invigoration-based Chinese medicine compounded formulas have significant anti-AS effects. In this study, we prepared Shoushen granule composed of Polygonum multiflorum, Wolfberry fruit, Raw hawthorn, and Notoginseng radix. In this Chinese herbal formula, Polygonum multiflorum is the major component to invigorate liver and kidney, benefit life essence, and nourish blood; Wolfberry fruit is an adjuvant herb to nourish liver and kidney, Notoginseng radix improves blood circulation to dissipate blood stasis, and Raw hawthorn is an adjuvant herb to promote Qi-flowing, dissipate blood stasis, and resolving turbidity, according to the theory of Traditional Chinese Medicine. This Chinese herbal formula invigorates kidneys, enriches essence, promotes blood circulation, and removes meridian obstruction. Our previous studies confirmed that Shoushen granule effectively regulate serum lipid levels in Carotid atherosclerosis (CAS) patients and ApoE−/− mice, and improves vascular elasticity in CAS patients, suggesting that Shoushen granule may have good therapeutic prospects in AS.

In conclusion, our findings suggest that a high-fat diet successfully induced AS formation in aortas and hyperlipidemia in ApoE−/− mice. Shoushen granule significantly alleviated the high-fat diet-induced AS pathological lesions in ApoE−/− mice and regulated lipid metabolism by increasing ABCA1 protein expression and serum IL-10 levels, and by downregulating PCSK9, TLR4, and NF-κB protein expression and serum TNF-α, MCP-1, and ICAM-1 levels. Combined with our previous findings, we believe that Shoushen granule may effectively regulate the expression of PCSK9 and the key factors in TLR4/NF-κB signaling path-
Figure 4 Effect of Shoushen granule on collagen content in mice

A1: aortic collagen content in Control group (x 40); A2: aortic collagen content in Control group (x 100); A3: aortic collagen content in Control group (x 200); B1: aortic collagen content in Model group (x 40); B2: aortic collagen content in Model group (x 100); B3: aortic collagen content in Model group (x 200); C1: aortic collagen content in Shoushen group (x 40); C2: aortic collagen content in Shoushen group (x 100); C3: aortic collagen content in Shoushen group (x 200); D1: aortic collagen content in Atorvastatin group (x 40); D2: aortic collagen content in Atorvastatin group (x 100); D3: aortic collagen content in Atorvastatin group (x 200); E: statistical chart of aortic Masson staining results. The aortic plaque Masson staining showed that the model group in the fiber plaque shaped cap, less matrix fiber content. Atorvastatin group increased and uniform fiber content in thin plaque matrix group and atorvastatin, Shoushen group and Atorvastatin group had no significant difference (P > 0.05). Data are presented as mean ± standard deviation, *P < 0.01, compared to Control group, Shoushen group and Atorvastatin group.

ways to promote ABCA1 expression, thereby achieving multi-targeted intervention of AS via lipid metabolism, inflammation, and immunity.

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Immunohistochemistry showed that the expression of ABCA4 was significantly decreased and the expression of PCSK9, TLR2 and TLR4 in Model group; D: NF-κB expression in Control group; B: NF-κB expression in Shoushen group; A: NF-κB expression in Atorvastatin group. Compared with Model group, the expressions of ABCA1 in Shoushen group and Atorvastatin group were significantly increased (P < 0.01). Shoushen group and Atorvastatin group had no significant difference (P > 0.05). Data are presented as mean ± standard deviation. *P < 0.01, compared to Control group, Shoushen group and Atorvastatin group.

Shoushen granule on arterial elasticity in patients with carotid atherosclerosis: a clinical randomized controlled trial. 


Shen DZ, Chen C, Chen JL, Xing SL. Effect of Shoushen...
Figure 6 Effect of Shoushen granule on the expression of ABCA1, PCSK9, TLR4 and NF-κB in mouse aorta
A: ABCA1, PCSK9, TLR4, NF-κB and β-actin expression exposed in Control group, Model group, Shoushen group, Atorvastatin group; B: statistical chart of ABCA1; C: statistical chart of PCSK9; D: statistical chart of TLR4; E: statistical chart of NF-κB. ABCA1: adenosine triphosphate binding cassette transporter A1; PCSK9: proprotein convertase subtilisin/kexin type 9; TLR4: toll-like receptor 4; NF-κB: nuclear factor kappa-B. Western blot showed that the expressions of ABCA1 were significantly decreased and the expression of PCSK9, TLR4, NF-κB was increased in Model group. Compared with Model group, the expressions of ABCA1 in Shoushen group and Atorvastatin group were significantly increased (P < 0.01), while the expression of Shoushen group, Atorvastatin group no significant difference (P > 0.05). Data are presented as means ± standard deviation. *P < 0.01, compared to Control group, Shoushen group and Atorvastatin group.


